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Determination of minor, trace and toxic elements in chewing tobacco products by instrumental neutron activation analysis and identification of glutamic acid

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Abstract Tobacco smoking/chewing has been a cause of concern because of it being related with oral cancer. It causes stimulation and ill physiological effects. Ten different brands of spit tobacco, eight gutkaas and five paan masalas have been analyzed for seven minor (Al, Na, K, Ca, Cl, Mg, and P) and 17 trace (As, Ba, Br, Co, Cr, Cs, Cu, Eu, Fe, Hg, La, Mn, Rb, Sb, Sc, Th, and Zn) elements by instrumental neutron activation analysis. Also Ni and Pb were determined by atomic absorption spectrophotometry. Concentration of Cd was below detection limit (<10 mg kg^{-1}) in the tobacco samples. Mg, generally added as $MgCO_3$ to prevent caking, is present as minor constituent in spit tobacco and gutkaas but is below detection limit (<1 g kg^{-1}) in paan masalas. Most elemental concentrations vary in a wide range depending on the nature of chewing tobacco. Spit tobacco has been found to be more enriched in essential elements (Ca, K, Na, P, Mn, and Rb), whereas gutkaas contain higher concentrations of Fe, Cr, Cu, and Zn. Paan masalas contain lower contents of other elements but higher content of Hg. Gutkaas also contain higher amounts of As and Pb. Further glutamic acid has been separated from tobacco leaves and characterized as it might bind with some elements.

Keywords Spit tobacco · Gutkaa · Paan masala · Elemental contents · INAA · Toxic elements

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Introduction

Tobacco is indigenous to many countries such as India, Middle–East, South-East Asia, and Americas, the name is derived from a Spanish/Caribbean word *tabaca* referring to the pipe or Y-shaped tube in which the broad, night shaded plant leaves were smoked. Botanically, tobacco is of the family *Solanaceae*, genus *Nicotiana.* Tobacco is generally used in two modes, smoking and chewing (non smoking), though a third mode called snuff tobacco is also prevalent in many countries. Abuse of tobacco by any means is a worldwide problem because of toxins including alkaloids and toxic heavy elements causing severe health effects [1]. It is chewed as betel, gutkaa, and zarda. Gutkaa and paan masala are processed products where some sweeteners and condiments including scented flavors are added. Gutkaa differs from paan masala in that the former contains more amount of tobacco and is relatively less processed whereas paan masala is more like a mouth freshener often taken after meals. Both are available in pouches made from aluminum foil and tin containers whereas chewing tobacco is mostly marketed in raw or shredded form. According to WHO survey [2], about 3 million deaths per year are caused worldwide by the consumption of tobacco. It increases the risk of oral cavity, cancer of the pharynx, larynx, and esophagus due to potent carcinogens including nitrosoamines, polycyclic aromatic hydrocarbons (PAH), toxic heavy metals, and radionuclides such as ^{232}Th and ^{210}Po.

A variety of tobaccos have been widely analyzed by different techniques such as atomic absorption spectrophotometry (AAS) [3], inductively coupled plasma-mass spectrometry (ICP-MS) [4], neutron activation analysis [5] and energy dispersive XRF spectrometry [6]. Recently Papastefanou [7] reported radioactivity due to the presence of naturally occurring radionuclides such as ^{226}Ra, ^{210}Pb, ^{137}Cs, and ^{40}K in tobacco leaves. Bagchi et al. [8] studied the cytotoxic effects of paan masala by the oxygen free radical interaction with membrane lipids and DNA leading to DNA damage and lipid peroxidation. In a recent study Waheed et al. [9] employed instrumental neutron activation analysis (INAA) for trace element evaluation in chewing tobacco and snuff. Verma et al. [10] determined trace element concentrations in smoking and chewing tobacco products and discussed the health implications. Looking at the importance of trace elements, two reference materials (RMs) of Oriental (OTL-1) and Virginia (VTL-2) tobacco have been developed by the Institute of Nuclear Chemistry and Technology (INCT), Poland [11].

It has been our observation that tobacco contains many toxic elements besides some nutrient elements [5]. Present study was undertaken to assess the minor, trace and toxic element contents in various brands of spit tobacco, gutkaas, and paan masalas, which are all chewed and swallowed. Ten different brands of spit tobacco, eight of gutkaas and five of paan masalas were analyzed for minor, trace and toxic elements by INAA. Furthermore, Pb and Ni were determined by AAS. An organic constituent glutamic acid was also isolated from the ethanolic extract of tobacco leaves and characterized by spectral studies so as to know the binding ligands with which these elements may be bound thus causing cancer.

Experimental

Sample collection and preparation

Different brands of spit tobacco, gutkaas, and paan masalas were procured from different cities of northern India. All the extraneous material was removed by hand picking and then surface contamination was wiped with tissue paper. The samples were oven dried at <80 °C, powdered in agate mortar and passed through 100-mesh sieve. A synthetic multielemental standard was prepared by depositing 2–5 μg amounts of As, Co, Cr, Fe, Sb, Se, and Zn salt (AR/HP grade) solutions in chloride/nitrate form on a Whatman filter paper strip. RMs of tobacco leaves (CTA-OTL-1 and CTA-VTL-2) from the INCT (Poland) and Apple Leaves (SRM 1515) from the NIST (USA) were dried before use.

Irradiation and counting

About 50 mg each of the powdered samples and RMs were weighed accurately and packed in alkathene/aluminum foil (Superwrap) for short (2 min)/long (3 days) irradiations, respectively, at $\sim 10^{13}$ cm^{-2} s^{-1} in CIRUS reactor at the Bhabha Atomic Research Centre (BARC), Mumbai. Short-lived activities were measured at the reactor site using an 80 cm^3 coaxial HPGe detector (EG & G ORTEC) and 4 k MCA. Long irradiated samples were brought to Roorkee after cooling for a month. γ-activity was measured using a HPGe detector having 1.8 keV resolution at 1,332 keV of ^{60}Co and 8k channel analyzer with GENIE-2000 software (Canberra, USA). Counting was followed at different intervals up to 3 months [5]. Phosphorus was determined by measuring β^- activity due to ^{32}P on an end window G.M. counter using 27 mg cm^{-2} Al filter as described earlier [12].

AAS measurements

Cd, Ni, and Pb were determined by AAS method using AAS (GBC Avanta, Australia). About 2 g of the powdered sample was weighed and dissolved in a (5:1 v/v) mixture of nitric and perchloric acids and repeated heating. After a clear solution was obtained, it was made up to 25 mL for analysis. Instrument was precalibrated using standard solutions of required concentration range.

Separation and identification of glutamic acid

50 g dry tobacco leaves were extracted with diethyl ether (8 h) in a Soxhlet to remove the tar. The extract was shaken up with 75 % aqueous ethanol, acidified with HCl to precipitate out resins and evaporated to $\sim$5 mL. The residue was hydrolyzed with 8 mL 6 M HCl at $\sim$100 °C for 2 h, filtered, washed, concentrated, and chromatographed using a mixture of benzene/methanol/acetic acid (45:8:2 v/v) on a 20 × 20 cm^2 TLC plate with 1-mm Silica Gel-G. Two bands were obtained at $R_f = 0.87$ and 0.76. The later band was scraped, dissolved in acetone, filtered, and distilled. 31 mg colorless crystalline compound so obtained was characterized by elemental analysis, infrared and NMR spectra including gas chromatography-mass spectrometry (GC–MS).

Results and discussion

Mean elemental concentrations ± SD for each brand were calculated using elemental standards and different RMs as

Table 1 Range and mean elemental contents in spit tobacco, gutkaas, and pan masalas

Element	Spit tobacco ($n = 10$)		Gutkaa ($n = 8$)		Paan masala ($n = 5$)	
	Range	Mean ± SD	Range mean ± SD	Mean ± SD	Range mean ± SD	Mean ± SD
Al (g kg^{-1})	0.48–3.46	1.50 ± 0.91	0.39–1.68	1.08 ± 0.42	0.13–1.67	0.70 ± 0.65
As (mg kg^{-1})	3.06–17.4	8.73 ± 5.37	3.97–26.9	19.3 ± 13.3	0.07–0.26	0.14 ± 0.07
Ba (mg kg^{-1})	25.9–63.8	42.8 ± 13.1	12.2–52.4	27.1 ± 14.4	2.37–10.2	6.07 ± 3.27
Br (mg kg^{-1})	8.40–88.9	54.3 ± 21.9	11.2–48.1	29.7 ± 11.6	4.80–19.6	11.5 ± 5.80
Ca (g kg^{-1})	23.5–55.8	36.9 ± 12.0	4.86–21.2	12.7 ± 5.16	5.39–17.6	10.9 ± 4.81
Cl (g kg^{-1})	0.63–2.20	1.16 ± 0.42	0.80–2.43	1.41 ± 0.63	0.78–2.55	1.32 ± 0.70
Co (mg kg^{-1})	0.25–1.04	0.67 ± 0.20	0.56–1.63	0.87 ± 0.38	0.08–0.13	0.10 ± 0.02
Cr (mg kg^{-1})	0.99–2.64	1.73 ± 0.48	1.75–3.44	2.87 ± 0.56	1.02–1.52	1.22 ± 0.22
Cs (mg kg^{-1})	0.17–0.39	0.28 ± 0.08	0.19–0.53	0.37 ± 0.11	0.17–0.24	0.19 ± 0.03
Cu (mg kg^{-1})	9.50–27.3	13.6 ± 5.57	9.87–20.3	14.7 ± 3.83	5.93–8.98	7.60 ± 1.13
Eu (μg kg^{-1})	15–51	29.7 ± 12.0	31.9–86.3	59.5 ± 18.1	18–42	27.2 ± 8.9
Fe (g kg^{-1})	0.26–1.80	1.17 ± 0.49	0.82–1.84	1.33 ± 0.35	0.12–0.18	0.16 ± 0.03
Hg (μg kg–^{1})	19–52	37.1 ± 10.5	51.1–116	74.2 ± 23.4	25.2–153	94.6 ± 57.9
K (g kg^{-1})	11.5–19.9	17.2 ± 2.39	1.98–10.4	6.37 ± 2.97	4.63–9.38	6.11 ± 1.94
La (mg kg^{-1})	1.13–2.13	1.61 ± 0.33	1.33–2.19	1.77 ± 0.29	1.15–1.31	1.43 ± 0.30
Mg (g kg^{-1})	3.26–16.7	6.59 ± 3.85	2.14–18.2	8.28 ± 5.04	<0.30	<0.30
Mn (mg kg^{-1})	96.4–212	132 ± 34	22.6–72.1	45.2 ± 18.6	18.7–82.2	46.3 ± 27.7
Na (g kg^{-1})	6.35–20.6	9.63 ± 4.04	0.29–4.97	1.75 ± 1.60	0.09–0.27	0.17 ± 0.08
Ni (mg kg^{-1})	0.16–1.10	0.85 ± 0.83	0.17–3.02	0.55 ± 0.31	0.04–0.12	0.09 ± 0.04
P (g kg^{-1})	2.34–3.20	2.91 ± 0.24	0.79–2.24	1.75 ± 0.51	1.05–1.44	1.25 ± 0.17
Pb (mg kg^{-1})	0.8–25.5	6.06 ± 7.05	0.67–34.1	10.9 ± 11.4	0.46–10.6	5.21 ± 4.29
Rb (mg kg^{-1})	24.9–33.1	29.0 ± 2.90	12.9–33.1	19.8 ± 6.98	8.82–21.1	14.7 ± 5.67
Sb (mg kg^{-1})	0.14–0.36	0.24 ± 0.08	0.13–0.22	0.16 ± 0.03	0.15–0.27	0.19 ± 0.04
Sc (mg kg^{-1})	0.15–0.40	0.29 ± 0.09	0.28–0.66	0.45 ± 0.13	0.03–0.05	0.04 ± 0.01
Th (mg kg^{-1})	0.04–0.08	0.06 ± 0.02	0.29–0.80	0.59 ± 0.16	0.03–0.05	0.04 ± 0.05
Zn (mg kg^{-1})	10.4–52.0	32.9 ± 12.5	44.0–84.9	60.6 ± 14.6	13.5–20.6	17.1 ± 3.40

comparators on the basis of replicate analyses, multiple photopeaks and counting at different intervals. A comparison of our data with the certified values for various RMs showed an excellent agreement within ±5 %. Hence we assume that our data for various brands of different samples should be accurate within 95 % confidence limit. Finally mean ± SD for each of three different products were calculated. ±SD was calculated for the number of samples analyzed in each group. Ranges and means along with ±SD for three different chewing tobacco products are listed in Table 1. Cd was found below detection limit (<10 μg kg^{-1}) and hence not reported. Significance of the elemental contents in chewing tobacco products is discussed below.

Elemental contents in spit tobacco

A perusal of ranges for elemental data in Table 1 shows that most elemental contents vary in a narrow range by a factor of 3, whereas Al, As, Co, Mg, Ni, and Zn exhibit large variations. However, Br (8.40–88.9 mg kg^{-1}), Fe (0.26–1.80 g kg^{-1}), and Pb (0.80–25.5 mg kg^{-1}) contents vary by an order of magnitude or even more. This could be attributed to geo-environmental factors and the soil characteristics where the tobacco plant was grown [13]. Particularly higher contents of Al, Ni, and Zn may be attributed to contamination from metallic pouches used as container. It is observed that spit tobacco which is least processed, is enriched in several nutrient elements such as Ca, K, Mg, P, and Fe.

Elemental contents in gutkaa

Ranges of elemental contents for eight different brands of gutkaas (Table 1) show that Na (0.29–4.97 g kg^{-1}), K (1.98–10.4 g kg^{-1}), Ni (0.17–3.02 mg kg^{-1}), and Pb (0.67–34.1 mg kg^{-1}) vary in a much larger range of the order of magnitude compared to all other elements though As (3.97–26.9 mg kg^{-1}) and Ca (4.86–21.2 g kg^{-1}) also vary significantly. Since gutkaas are processed tobacco products, variation in elemental contents could be due to different additives and industrial processing like pre-harvest and post harvest operations, pre-blend operations, drying, storing, processing, and flavor addition [6]. Such intra brand variations due to physical and chemical processing may be attributed to different processing technologies which are exclusive and different for each brand [10].

Elemental contents in paan masala

Paan masalas contain very little tobacco and more of other ingredients such as shredded areca nut, catechu, cardamom, mint, and some sweetener. These are more processed compared to gutkaas. It is observed that most elemental contents vary in a narrow range except Al (0.13–1.67 Mg kg^{-1}), Hg (25.2–153 μg kg^{-1}), and Pb (0.46–10.6 mg kg^{-1}). It seems that some contamination may be occurring from containers during processing. There has been a controversy regarding addition of $MgCO_3$ to chewing tobacco products so as to prevent caking [14]. $MgCO_3$ is considered as a potent carcinogen, which has prompted many state governments in India to ban its use. Role of magnesium in aging, preterm labor, cardiovascular mortality and its correlation with toxic metals has been discussed in [15]. It has been shown that cytotoxic effects of chewing paan masalas are mediated through the production of reactive oxygen species (ROS) [8]. Further, some metal ions such as Cr, Mn, and Zn may play role in the production of ROS thereby may be causing oral cancer [16].

Comparison of mean elemental contents in different chewing products

Mean contents of minor and toxic elements in three different varieties of chewing products have been compared as bar plots in Figs. 1, 2, respectively. A perusal of data in Table 1 and the figures shows that most elemental contents in spit tobacco are higher except Mg, Fe, Cr, Cu, and Zn, whereas toxic elements As, Hg, and Pb are lower. On the other hand, most element contents in paan masalas are lower. Thus, spit tobacco or raw form of tobacco is most enriched in Ca, K, Na, P, Mn, and Rb all of which are essential for biochemical processes [17]. Particularly higher Na content in some samples could be attributed to deliberate addition of common salt to suppress the acrid taste as well as a preservative to prevent biodegradation. This form of tobacco is very popular in rural areas and among low-income groups where it provides stimulation for heavy manual work. Spit tobacco also contained higher amounts of Cr, Fe, Cu, and Zn, known for their anti-oxidant behavior [16]. In general, paan masalas contain least amounts of Ca, Fe, K, Na, P, Cr, Cu, Mn, Rb, and Zn, all nutrient elements but higher amount of Hg (still <permissible level of 0.5 mg kg^{-1}) which may be responsible for its oral cancer causing effects. It is observed that mean contents of As and Pb the most toxic elements are highest in gutkaas. Although the global daily dietary intake of As and Pb from food are 12 and 16 mg kg^{-1}, respectively, a moderate gutkaa user consuming 50 g pouch per day may ingest much higher amounts of As and Pb [18]. Pb contents in all three varieties of tobacco do not vary significantly, though paan masalas contain the least amounts of As and Ni. Cadmium another heavy toxic metal present in cigarette tobacco and ash was found lower than detection limit (<10 μg kg^{-1}) in all the three varieties of chewing tobacco. It is to be noted that spit tobacco has higher Ni content. However, contents of all toxic elements except Pb (in some cases where it is >10 mg kg^{-1}) are

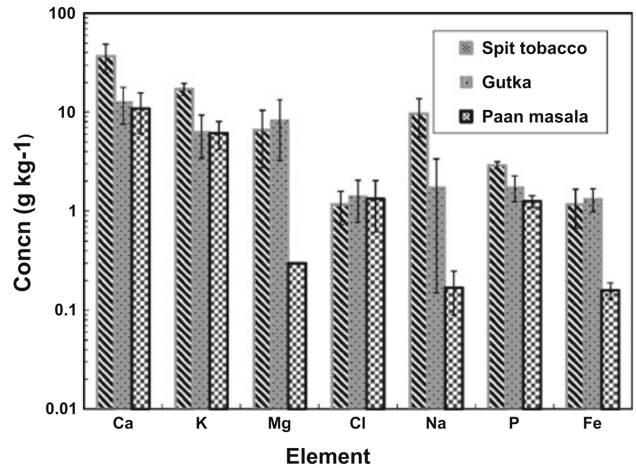


Fig. 1 Variation in minor element concentrations in different chewing tobacco products

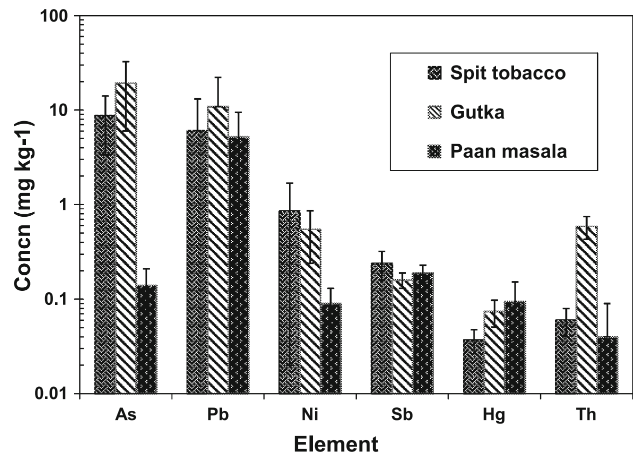
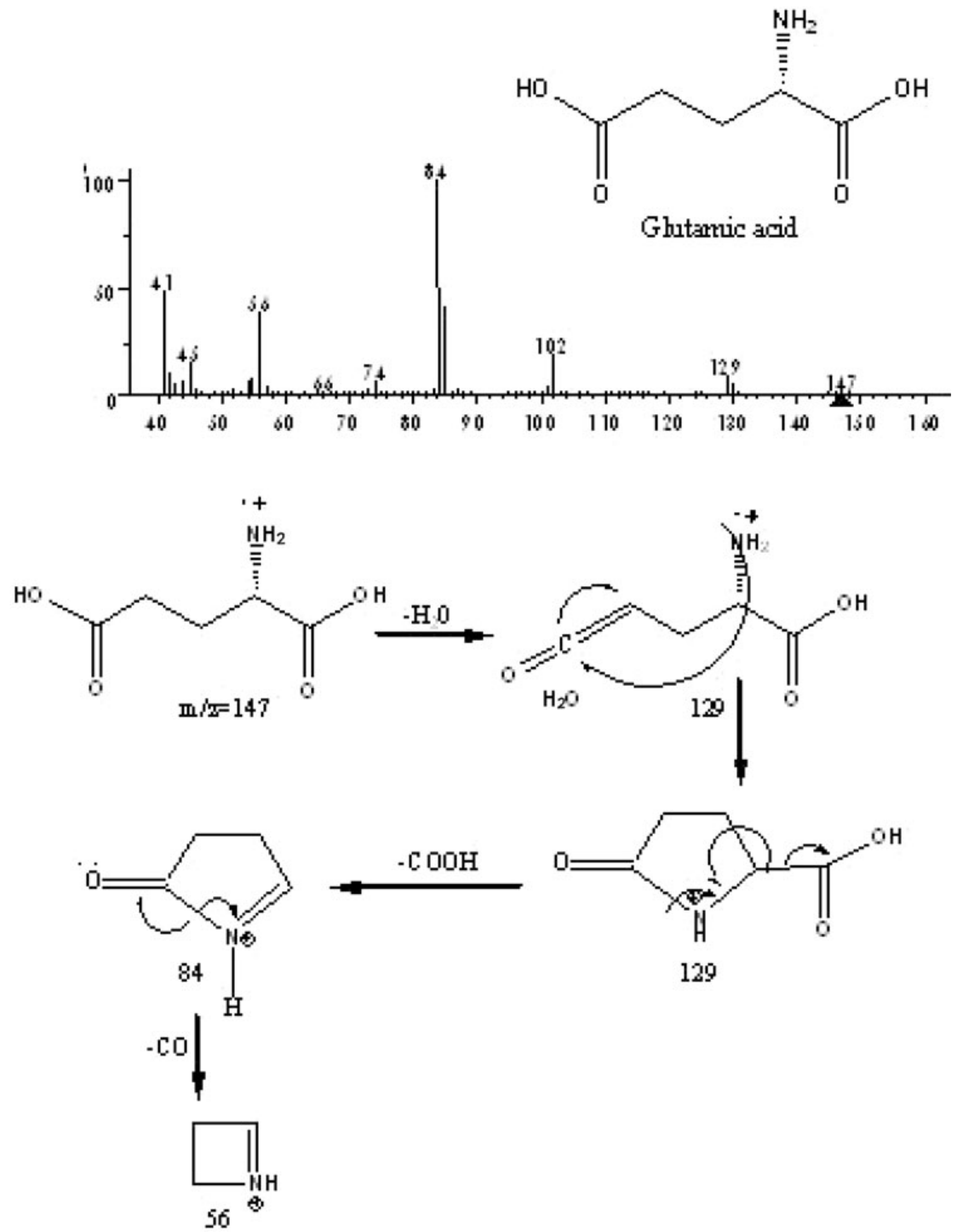


Fig. 2 Variation in toxic element concentration in different chewing tobacco products

Fig. 3 Mass spectrum and suggested fragmentation pattern of glutamic acid



well below the permissible limits [19]. Waheed et al. [9] have reported significant amounts of Pb and Cd in chewing tobacco from Pakistan. The adverse health effects of toxic elements on the fetus through parental chewing of tobacco especially of gutkaas is of much concern as the fetus and young children are particularly sensitive to heavy toxic elements [20].

K and P were found to be linearly correlated ($r = 0.93$) in spit tobacco but no such correlation was observed for gutkaas and paan masalas. K/P value is found to be highest (5.90 ± 0.45) in spit tobacco compared to that in gutkaas (3.67 ± 1.29) and paan masalas (4.90 ± 1.38). On the basis of elemental contents, it may be concluded that chewing of gutkaa and paan masala is more harmful as far as toxic element contents are concerned. However, spit tobacco provides several nutrient elements as well besides toxic elements.

Identification of glutamic acid

It is a colorless crystalline solid ($C_5H_9NO_4$), yield = 230 mg kg^{-1}, mp: 260° (lit. mp 262.1°), $R_f = 0.76$ in benzene/methanol/acetic acid in 45:8:2 v/v: Elemental contents (%): C, 41.14 (40.82); H, 6.32 (6.32); N, 9.09 (9.52) where values in brackets are calculated; IR (cm^{-1}) in KBr: 2,650–3,350 (super imposition of ν_{O-H} with ν_{N-H}), 2,128 (overtone due to asymmetric δ_{N-H} and the torsional oscillation of the $-NH_3^+$ group), 1,657 (asymmetrical δ_{N-H}), 1,585 (symmetrical $\delta_{N–H}$), 1,271($v_{C–O}$); ^{1}H NMR in d_6 DMSO (δ in ppm) 11.0 (O–H in carboxylic acid), 3.47 (C*H–NH_2), 2.51 (–CH_2–C*H_2COOH), 2.34 (–C*H–CHR–NH_2), 1.9 (NH_2); ^{13}C NMR in d_6 DMSO (δ in ppm), 176, 173 (C of two carboxylic acid groups), 54 (*C–NH_2), 38 (*CH_2–COOH), 30 (–*CH_2–CH_2–COOH); MS at $R_t = 15.3$ m/z (rel. int.) 129 (3.56), 84 (100), 56 (21.4) [21]. Mass spectrum of the compound and possible fragmentation pattern, shown in Fig. 3, confirm the presence of glutamic acid. Glutamic acid, widely distributed in proteins helps nourish the body but free glutamic acid as monosodium glutamate (MSG) shows debilitating and life threatening effects by stimulating brain neurons [22].

Conclusion

INAA and AAS study of 27 elemental contents in three forms of chewing tobacco (ten brands of spit tobacco, eight of gutkaas, and five of paan masalas) show that spit tobacco and gutkaa are a rich source of Na, K, P, and Fe, but paan masala contained least amounts of these elements. K and P exhibit linear correlation ($r = 0.93$) in spit tobacco with highest K/P value compared to gutkaas and paan masalas. Mg was not detected in paan masala though it is present as a minor constituent in spit tobacco and gutkaas. Anti-oxidant elements (Cr, Mn, Fe, Cu, and Zn) are also present in elevated amounts in spit tobacco compared to that in gutkaa*s* and paan masalas where these act as co-carcinogen. Pb, a heavy toxic metal is present in significant amounts ($\sim$10 mg kg^{-1}) in all the three tobacco products. Glutamic acid was isolated from raw tobacco and was characterized. It may act as a potential ligand for binding various metal ions.

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